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H. 知的財産権の出願・登録状況

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7. その他

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厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）
（分担）研究報告書

心筋幹細胞移植実現化へ向けての最適な血管新生療法探索研究に関する研究

（分担）研究者　尾池　雄一　　熊本大学大学院医学薬学研究部教授

研究要旨

重症心不全への心臓移植事業がドナー不足のため充分には機能していない現状を打開することを目的とする末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成研究の中で、私は細胞移植による心筋・血管再生医療が実現化されるために最も適した血管を誘導できる新生方法の確立、血管新生因子の探索と同定に関する研究を行う。

尾池雄一・熊本大学　教授

C. 研究目的

重症心不全への心臓移植事業がドナー不足のため充分には機能していない現状を打開することを目的とする末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成研究の中で、私は細胞移植による心筋・血管再生医療が実現化されるために最も適した血管を誘導できる新生方法の確立、血管新生因子の探索と同定に関する研究を行う。

B. 研究方法

アンジオポエチン様血管新生因子ファミリー(Angptl)の血管新生因子としての機能解析は、各々の遺伝子改変マウスを作製し機能解析を行った。さらに臨床応用に向けて各々の組み替えタンパクの作成や発現調節機構の解析を進めている。

（倫理面への配慮）

動物操作にあたっては各施設の動物実験指針に従って行う。基礎的研究においては、遺伝子改変マウス、プラスミドDNAを用いる場合は仕様に際しては、遺伝子組み換え生物などの使用等の規則による生物の多様性の確保に関する法律に基づき研究を実施する。今後の臨床研究に関しては、慶應義塾大学医学部の倫理委員会承認のもとで進め、患者、ボランティアに対するインフォームド・コンセントは書面で行う。

C. 研究結果

AGF/Angptl6は血管新生作用のみならず、上皮細胞への増殖作用も有し、注入される細胞にとっての足場形成には適した分子で

あることを明らかにしてきた。さらに、重症新心不全の原因となる重篤な虚血性心疾患の基盤病態と言えるメタボリックシンドロームに対しても治療効果を有していることを明らかにし、単に心筋幹細胞移植時に最適な血管新生療法を行える分子としてのみならず、その基盤となる病態改善にも寄与できる可能性を明らかにしてきた。そこで本年度はマウス下肢虚血モデルを用いてAGF/Angptl6が実際に血管再生、血流改善できるかを検討し、充分に血流を改善でき、虚血による下肢の切断イベントを抑制できることを見出した。また、その機構として血管新生のみならず副側血行路を増やす作用によること、分子機構としてERK1/2-eNOS-NO経路を活性化させることを見出した。また、候補として考えていたAngptl2は、炎症病態で認められるような病的な血管新生を誘導することを見出し治療的血管新生には不適であることを見出した(論文準備中)。心筋・血管再生医療における再生心筋および血管細胞の供給源として骨髄細胞が重要であるが、生体内で増幅できないかどうか細胞周期関連分子のたんぱく質分解を介して細胞周期を抑制的に制御するユビキチンリガーゼFbxw7の骨髄細胞特異的遺伝子欠損マウスを作製し検討した。Fbwx7が欠損すると高率に白血病を発症するとともに、骨髄幹細胞を細胞周期の静止期にとどまることができずに過剰な細胞分裂の結果、骨髄幹細胞が枯渇してしまうことを明らかにした。

D. 考察

欠損した組織で注入された細胞が増殖し組織を再生誘導するためには、適切な細胞増殖因子と細胞周囲の環境が重要であるが、AGFは血管新生作用のみならず上皮細胞への増殖作用も有し注入される細胞にとっての足場形成には適した分子であること、AGFが同時に重症新心不全や細胞移植治療後の再発の原因となる重篤な虚血性心疾患の基盤病態と言えるメタボリックシンドロームに対しても治療効果を有していること、に加えて本年度にAGFが実際にマウスの虚血病態で血流を改善でき壊死から回避できることを明らかにしたことは心筋幹細胞移植による心不全への細胞移植事業実現化へ向けて最も適した血管を誘導できる方法を探索し検証するという点において多大に貢献したものと考えられる。一方で心筋・血管再生医療における再生心筋および血管細胞の供給源である骨髄幹細胞を細胞周期制御因子の調節により生体内で増やすことは様々な予期せぬ副作用を誘発すると考えられた。

E. 結論

心筋幹細胞移植による心不全への細胞移植事業実現化へ向けて最も適した血管新生療法の開発にAGF/Angptl6が貢献できる可能性,を見出した。一方で候補として考えていたAngptl2は、炎症病態で認められるような病的な血管新生を誘導することを見出し治療的血管新生には不適であることを見出した。再生心筋および血管細胞の供給源である骨髄幹細胞を生体内で増やすことは

失敗した

F. 健康危険情報

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研究成果の刊行に関する一覧

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Skeletal muscle-derived progenitors capable of differentiating into cardiomyocytes proliferate through myostatin-independent TGF-β family signaling

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Abstract

The existence of skeletal muscle-derived stem cells (MDSCs) has been suggested in mammals; however, the signaling pathways controlling MDSC proliferation remain largely unknown. Here we report the isolation of myosphere-derived progenitor cells (MDPCs) that can give rise to beating cardiomyocytes from adult skeletal muscle. We identified that follistatin, an antagonist of TGF-β family members, was predominantly expressed in MDPCs, whereas myostatin was mainly expressed in myogenic cells and mature skeletal muscle. Although follistatin enhanced the replicative growth of MDPCs through Smad2/3 inactivation and cell cycle progression, disruption of myostatin did not increase the MDPC proliferation. By contrast, inhibition of activin A (ActA) or growth differentiation factor 11 (GDF11) signaling dramatically increased MDPC proliferation via down-regulation of p21 and increases in the levels of cdk2/4 and cyclin D1. Thus, follistatin may be an effective progenitor-enhancing agent neutralizing ActA and GDF11 signaling to regulate the growth of MDPCs in skeletal muscle.

Keywords: Muscle progenitor cells; Follistatin; Proliferation; Myostatin; TGF-β

Cardiac myocytes undergo terminal differentiation and lose the ability to divide soon after birth, withdrawing irreversibly from the cell cycle. Thus, the loss of myocytes after myocardial infarction results in irreparable damage to the adult heart, ultimately leading to eventual heart failure. A growing number of studies focused on cell-based therapy have demonstrated the isolation of adult stem cells from a variety of tissues and shown their ability to promote cardiac repair, but the task of inducing myocyte repopulation is fraught with obstacles [1].

Adult skeletal muscle harbors a pool of satellite cells associated with single myofibers, which possess remarkable regenerative potential as endogenous muscle progenitors [2]. Although these progenitor cells preferentially generate differentiated cells of the same type as cells in their tissue of origin, several studies have indicated that at least a fraction of stem cells from skeletal muscle, termed muscle-derived stem cells (MDSCs), can generate cells of different lineages [3] and may be much more plastic than previously appreciated. Recent reports demonstrated that Pax7-expressing muscle-derived subpopulation that could be isolated from suspended clusters of cells (myosphere) may contain a heterogeneous subpopulation capable of differentiating into myogenic, osteogenic, and adipogenic lineages; however, their potential of cardiac differentiation has yet to

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be determined [4]. In addition, clonal analysis of MDSCs isolated from neonatal skeletal muscle demonstrated that they constituted a subset of a Sca-1-expressing cell population [5]. However, an additional study in the adult mice confirmed that Sca-1 negative cell population, termed skeletal-based precursors of cardiomyocytes (Spoc), also contained cells that were able to differentiate into cardiac muscle cells [6], suggesting the heterogeneity in the pool of cardiogenic progenitors within MDSCs. To study the functional properties of muscle-derived progenitor cells, we prospectively isolated progenitor clones from adult skeletal muscle, based on the characteristics of adult stem cells having a distinct proliferative potential to form floating-spheres, termed myospheres [7].

The maintenance of muscle-derived stem/progenitor cells is controlled by growth factors and cytokine signals [8,9]; however, few studies have explicitly investigated the factors contributing cardiogenic progenitor cells from adult skeletal muscle to proliferate in vitro and in vivo. In the present study, we identified follistatin, an activin-binding protein to inhibit TGF-β family members, as a direct modulator of cardiogenic progenitor cell proliferation from adult skeletal muscle. ActA and GDF11, capable of binding the activin type II receptors (ActRII) [10,11], are the potent negative regulators of MDPC activation. These results may greatly facilitate the process of cardiogenic-progenitor cell ex vivo expansion as well as the development of new transplantation technologies for regenerative medicine.

Materials and methods

Mice. The *mstn−/−* mice were provided by S.-J. Lee (The Johns Hopkins University) [12]. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Kyoto University.

Progenitor cell isolataion and differentiation. Primary hind limb muscle cells were isolated from 6- to 12-week-old C57BL/6J (Shimizu Laboratories Supplies), *mstn−/−*, or *mstn+/+* littermate mice using 15 mg collagenase type II/g tissue for digestion (Worthington). Skeletal myospheres were isolated and expanded as previously described [7]. Cells were suspended in serum-free medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27 (Invitrogen), 20 ng/ml mouse EGF (SIGMA), 40 ng/ml human recombinant bFGF (Promega), and 1% penicillin/streptomycin. Single-cell suspensions were then preplated on non-coated cell culture plates (Corning) at 20 cells/μl density for 7 days to obtain myogenic cells. Cells proliferated to form floating myospheres in cell suspension after 7 days and were selectively picked from the plates and transferred into a fresh fibronectin-coated culture plates for adherent culture. For myogenic cell isolation, the cells that adhered within first 7 days were passaged to remove fibroblasts, and then the culture was enriched with satellite cells and myoblasts. The cell growth medium was replaced with MEM, 10% FBS, insulin–transferrin–selenium supplements (Invitrogen), and 10^{-8} M dexamethasone (SIGMA) for cardiac differentiation. The attached cells, during preplating cell-culture, containing myogenic cells were maintained in the same growth medium for further expansion. C_2C_{12} myoblasts and mouse ES cells were maintained as previously described [7,13].

RT-PCR and Gene expression analysis. Total RNA was extracted using TRIzol Reagent (Invitrogen) and first-strand cDNA was synthesized using a SuperScript III kit (Invitrogen). PCR reactions were performed with gene-specific primers for 30–35 cycles. The gene-specific primer sequences are shown in Supplementary Table.

Electrophysiology. Three days before electrophysiological experiments, the cells were transfected using polyethyleneimine with a plasmid expressing EGFP under the control of the α-MHC promoter to visualize differentiated cardiomyocytes [13]. For membrane potential measurements, current-clamp experiments were performed at 37 °C, using the whole-cell configuration of the patch-clamp technique (Axopatch 200A; Axon Instruments) [14].

Immunofluorescence. Cells were fixed in 4% paraformaldehyde and were stained with mouse monoclonal antibodies against cardiac troponin-T and cardiac troponin-I (Hytest). Secondary antibodies were conjugated with Alexa Fluor 488 or Alexa Fluor 555, and nuclei were visualized using DAPI (all from Molecular Probes). Images were captured with a BZ-8000 microscope (Keyence, Japan).

Construction of knockdown vectors. The mouse ActA and GDF11 gene sequences were analyzed for potential siRNA targets using the web-based siRNA target finder (Clontech website). The siRNA target sequences accordingly designed to suppress ActA and GDF11 were as follows: ActA siRNA-1: CTTGCTTTGGCTGAGAGGATT; ActA siRNA-2: CGA AATGAATGAACTCATGGA; ActA siRNA-3: TCTTTCCAGTGTC-CAGCAGCA; GDF11 siRNA-1: TCCTCTCACAGTGGACTTTGA; GDF11 siRNA-2: GCGAATACATGTTCATGCAAA; GDF11 siRNA-3: CAATGACAAGCAGCAGATTAT. Each target sequence was subcloned into the BamHI-EcoRI site of RNAi-Ready-pSIREN-RetroQ vector (Clontech) as an inverted repeat with a hairpin loop spacer. RNAi-Ready-pSIREN-RetroQ-luciferase vector was used as a Mock vector.

Retrovirus production and infection. GP2-293 cells (Clontech) were cotransfected with the envelope vector pVSV-G and retrovirus vectors encoding the puromycin resistance gene using FuGENE6 (Roche). MDPCs were infected with retrovirus in the presence of 4 μg/ml polybrene for 24 h, and the infected cells were selected with 2.5 μg/ml puromycin.

Flow cytometric analysis (FACS). One hundred thousand cells were seeded into a 60-mm culture plate and incubated for 24 h in growth medium. After 24 h of serum and growth factor deprivation to synchronize the cell cycle, the MDPCs were re-stimulated with serum-containing medium for a further 12–16 h. Cell cycle phase was studied using a flow cytometer (FACSCalibur, BD Biosciences) for at least 30,000 individual events per reaction. Data were analyzed using Mod-fit software (Verity Software House, Inc.).

Western blotting. MDPCs were extracted with lysis buffer containing 50 mM Tris–HCl, (pH 7.6), 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, and a protease inhibitor cocktail Kit. The transferred membranes were incubated with mouse monoclonal antibodies against p21, phospho-cdk2/4, cyclin D1/E (Santa Cruz), ActA (R&D) or GAPDH (CHEMICON); goat polyclonal anti-GDF11 (Santa Cruz); or rabbit polyclonal antibodies against phospho-Smad2/3, phospho-Smad1/5/8 (Cell Signaling), Smad2/3, or Smad1/5/8 (Upstate).

Statistical analysis. Data are means ± SE and were analyzed by ANOVA and Scheffe's test, using a significance level of $p < 0.05$ (StatView).

Results

Myospheres contain progenitor cell able to differentiate into functional cardiomyocytes

To determine whether myospheres could generate cardiac muscle cells, we isolated the individual spheres from floating culture as previously described [7]. For myosphere expansion, individual spheres were transferred onto fibronectin-coated 24-well plates with 2% serum-containing medium and the spheres were allowed to attach. Myospheres could be repeatedly formed, as secondary spheres, from the adherent progeny of primary spheres that had been passaged one time. Cardiac differentiation was documented at 10 days after induction with 10^{-8} M dexameth-

asone as verified by cardiac troponin-T (Fig. 1A; $1.4 \pm 0.3\%$, $n = 12$) and cardiac troponin-I staining (Fig. 1B; $1.7 \pm 0.3\%$, $n = 10$). To monitor differentiated cardiomyocytes more carefully, we constructed a vector in which cardiac-specific α-myosin heavy chain (α-MHC) promoter drives expression of an EGFP reporter cDNA. Transduction of MDPCs with the α-MHC-EGFP plasmid enabled visualization of differentiated cardiomyocytes as live cells (Figs. 1C and D). Fully differentiated cardiomyocytes expressed connexin-43/45 as well as the L-type calcium channel Cav1.2 in monolayer cell culture (Fig. 1E). The expression of cardiac differentiation markers, including cardiac transcription factors, structural proteins, and natriuretic peptides under identical differentiation conditions, was induced, as verified by RT-PCR (Fig. 1F).

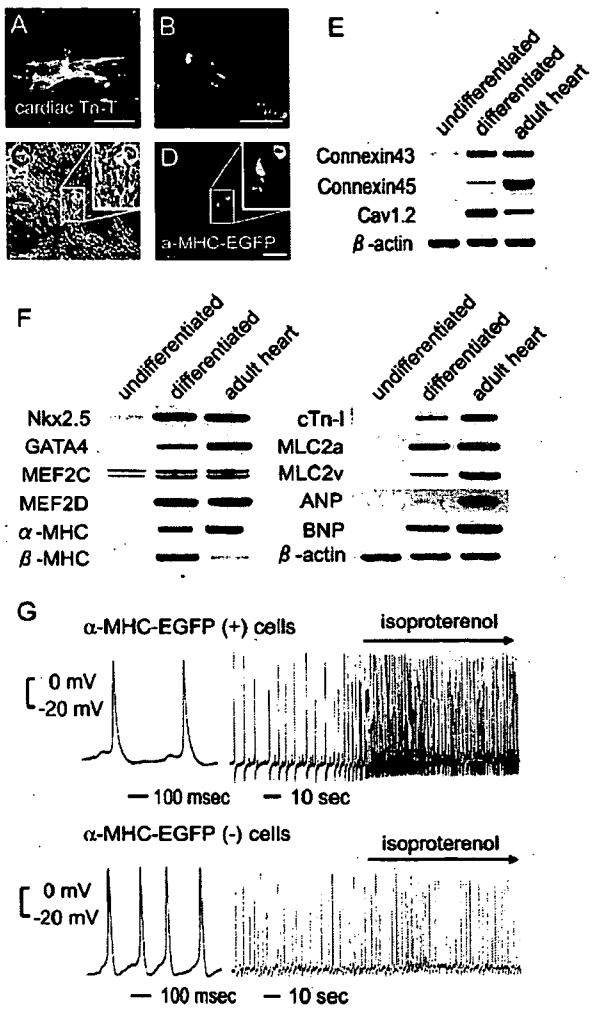

Fig. 1. Myospheres contain progenitor cells able to differentiate into functional cardiomyocytes (A and B) Myosphere-derived cells were treated with dexamethasone for 10 days and gave rise to cardiomyocytes. Cultures were stained for cardiac troponin-T (A, green) and cardiac troponin-I (B, red) to verify cardiac differentiation. DAPI, blue. (C and D) α-MHC promoter-driven EGFP plasmid was transfected into adherent progeny of MDPCs at day 7 during cardiac induction. Differentiated cardiomyocytes at day 10 are shown by phase contrast (C) and green fluorescence (D). Scale bars represent 20 μm. (E) RT-PCR showed the expression of gap junction proteins and L-type calcium channel Cav1.2 in differentiated cardiomyocytes. (F) RT-PCR for cardiac transcription factors (Nkx2.5, GATA4, MEF2C, and MEF2D) and structural genes (αβ-MHC, cTn-I, MLC2a/2v, ANP, and BNP) in differentiated and undifferentiated MDPCs. Two-month-old adult hearts were used as a positive control. (G) Action potential analysis. APs obtained from differentiated cardiomyocytes, verified as α-MHC-EGFP$^+$ cells responded to 10^{-7} M isoproterenol treatment (upper). APs acquired from non-cardiac muscle cells lacking EGFP fluorescence were not affected by 10^{-7} M isoproterenol treatment (bottom). Data shown are representative results obtained from 5 independent experiments.

To further characterize the differentiated cells from the adherent progeny of MDPCs, we then mapped the optimal action potentials (APs), focusing on EGFP$^+$ cardiomyocytes (Fig. 1G) or EGFP$^-$ non cardiac-cells under the spontaneous beating-condition after transfection of α-MHC-EGFP plasmid into undifferentiated MDPCs. Differentiated cardiac muscle cells contracted spontaneously at a lower rate than EGFP$^-$ cells (147 ± 14.7 vs 309 ± 23.3 beats per minute; $p < 0.01$). Although the depolarization amplitude was similar between EGFP$^+$ and EGFP$^-$ cells (66.6 ± 5.7 vs 68.8 ± 4.2 mV; n.s.), the resting membrane potential in EGFP$^+$ cardiomyocytes was higher than that in EGFP$^-$ cells (-46.6 ± 4.2 vs -54.6 ± 3.9 mV; $p < 0.05$). We next sought to determine whether the differentiated cardiomyocytes have β-adrenergic receptors, as assessed by their response to the β-adrenergic agonist isoproterenol. Indeed, the cardiac beating rate increased significantly in EGFP$^+$ cardiomyocytes in response to 10^{-7} M isoproterenol stimulation (Fig. 1G), whereas EGFP$^-$ non-cardiac cells contracted with irregular rhythm and showed a lack of increase in AP frequency.

Follistatin is a sphere-derived intrinsic factor that increases the proliferative potential of myospheres independent of myostatin

To investigate the molecular mechanisms of the MDPC proliferation, the expression patterns of myostatin, a secreted growth and differentiation factor (GDF8), and its antagonist follistatin were examined [15]. We found that follistatin was highly expressed in myospheres and ES cells, and was expressed to a lower extent in myogenic-lineage committed cell types (Fig. 2A). In contrast, myostatin expression was neither detectable in primary nor secondary myospheres but was abundant in myogenic cells and skeletal muscle tissue.

We examined the down-stream targets of TGF-β family signaling and cell cycle regulators in response to follistatin treatment. Follistatin inhibited Smad2/3 phosphorylation with a reciprocal increase in Smad1/5/8 phosphorylation. The effects of follistatin on MDPCs were characterized by a decrease in p21 expression and an increase in cdk2 activation (Fig. 2B). We next asked if the increased proliferation observed in follistatin-treated MDPCs is due to deregulation of the G1 to S phase transition. FACS analysis of the cell cycle distribution of a synchronous cell population revealed that follistatin significantly enhanced the

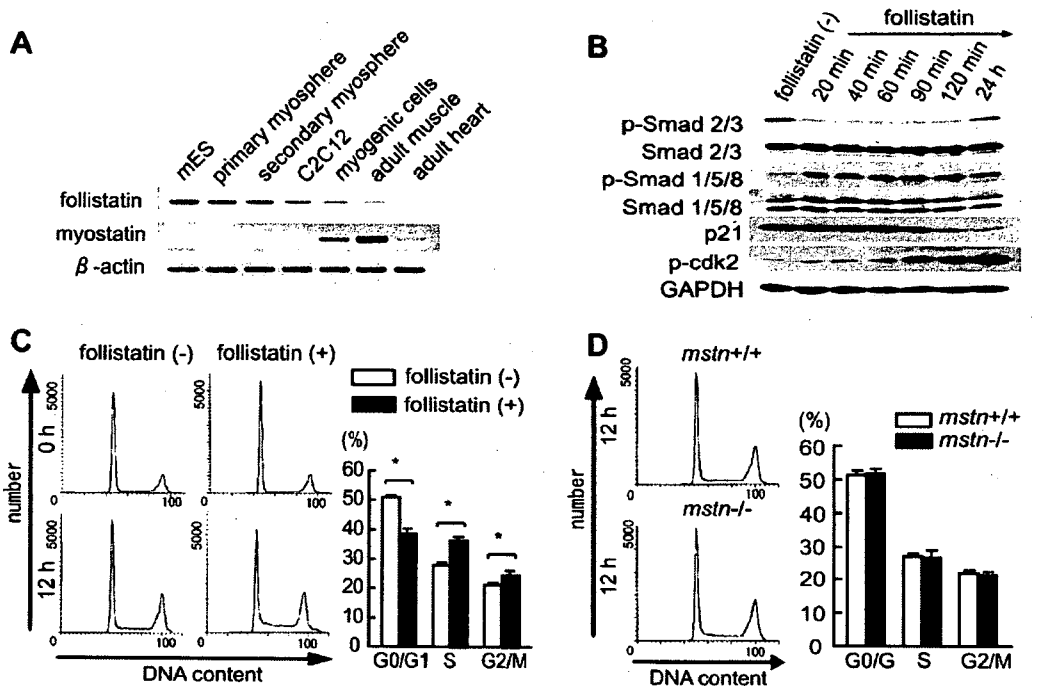

Fig. 2. Intrinsic myostatin is not the follistatin-sensitive ligand to regulate the growth of MDPCs through inhibition of Smad2/3 phosphorylation. (A) RT-PCR for myostatin and its antagonist, follistatin, in myospheres. (B) Actively proliferating myospheres treated with follistatin were characterized by the inhibition of Smad2/3 phosphorylation, down-regulation of p21, and activation of Smad1/5/8 and cdk2. (C) FACS analysis. MDPCs were cultured with or without follistatin (20 nM) for 7 days followed by 24 h serum-free starvation. The cells were then harvested at 0 and 12 h after cell culture in growth medium and subjected to flow cytometry analysis ($n = 5$). G0/G1, S, and G2/M phases of cell cycle for each treatment are summarized on the right. $^{*}p < 0.01$. (D) FACS analysis. MDPCs were isolated from *mstn*−/− or *mstn*+/+ mice and were grown in the growth medium for 12 h before harvesting. Cell cycle distribution is summarized on the right ($n = 5$).

cell cycle progression in MDPCs cultured in growth medium for 12 h (Fig. 2C).

Since it is known that follistatin inhibits the binding of myostatin to ActRII, we next focused on myostatin for further investigation because of its potential role in negative regulation of skeletal muscle growth [12,15]. For this purpose, myospheres were generated using the cells isolated from myostatin null (*mstn*−/−) and from age-matched littermate controls (*mstn*+/+) and examined the cell cycle distribution of the MDPCs. We subjected synchronous, actively proliferating MDPCs cultured in growth medium for 12 h to FACS analysis and found that MDPCs isolated from *mstn*−/− and littermate *mstn*+/+ mice had a similar cell cycle progression profile from mitosis through G1 to the next S phase (Fig. 2D).

ActA and GDF11 negatively regulate MDPC growth

Because Smad1/5/8 are thought to be specific for bone morphogenetic protein (BMP) 2/4/7 signaling [16], we next investigated whether endogenous activin and TGF-β family signaling that might be blocked by follistatin and might regulate myosphere proliferation via Smad2/3 phosphorylation [10,11,16]. RT-PCR analysis showed that myospheres were positive for activin A/B and GDF11 but not nodal expression (Fig. 3A), suggesting that the follistatin-induced inhibitory interaction of ActRII to activin and GDF11 binding might produce the rapid progression of myospheres.

To directly test whether the ActA and GDF11 signaling is required for the MDPC development, we targeted endogenous expression of ActA and GDF11 by using siRNA technology. We first assessed the efficacy of the siRNAs by testing their ability to inhibit endogenous protein levels of ActA and GDF11. The specificity of the siRNAs for ActA and GDF11 was verified by the use of uninfected MDPCs and Mock controls (Figs. 3B and C). The most effective siRNA for each was selected for use in the following experiments. MDPCs infected with siRNA of either ActA or GDF11 showed a significant increase in the replicative potential during 21 days in culture, in comparison to the Mock control (Fig. 3D).

Knockdown of ActA and GDF11 accelerates cell cycle progression in MDPCs

To quantitate the effects of ActA and GDF11 on the MDPC development, we next carried out FACS analysis. We synchronized the MDPCs in G0 phase by culturing the cells in serum-free conditions. When MDPCs were stimulated by serum treatment for 16 h, 46.3 ± 0.4% and 52.1 ± 0.8% of control and GDF11-silenced MDPCs,

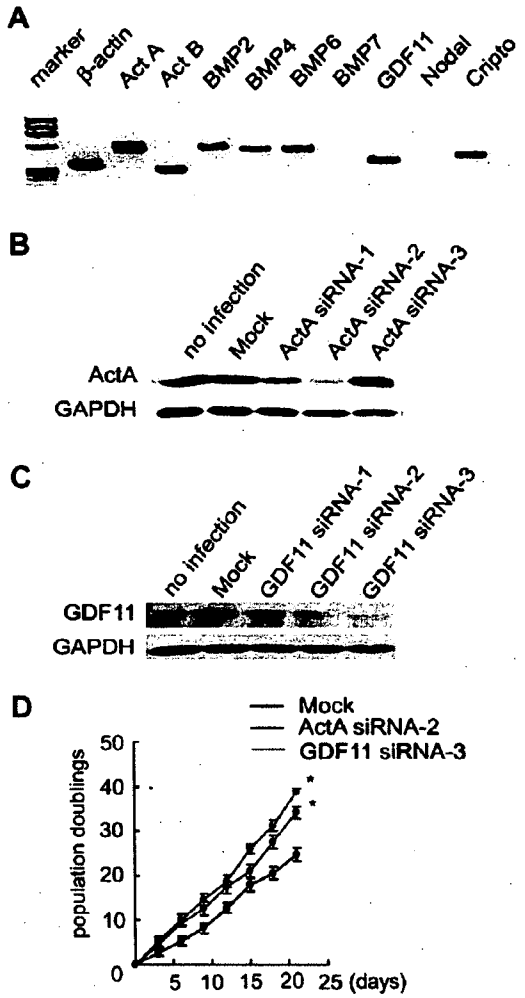

Fig. 3. ActA and GDF11 negatively regulate MDPC growth. (A) RT-PCR for the expression of ligands in activin and TGF-β family signaling. Protein levels of ActA (B) and GDF11 (C) following specific-siRNA infection. Three different siRNAs, as indicated, were tested for each. (D) Growth kinetics of MDPCs infected with the respective siRNAs as indicated. Data were obtained for 3 independent clones from MDPCs treated with each siRNA. $^{*}p < 0.01$.

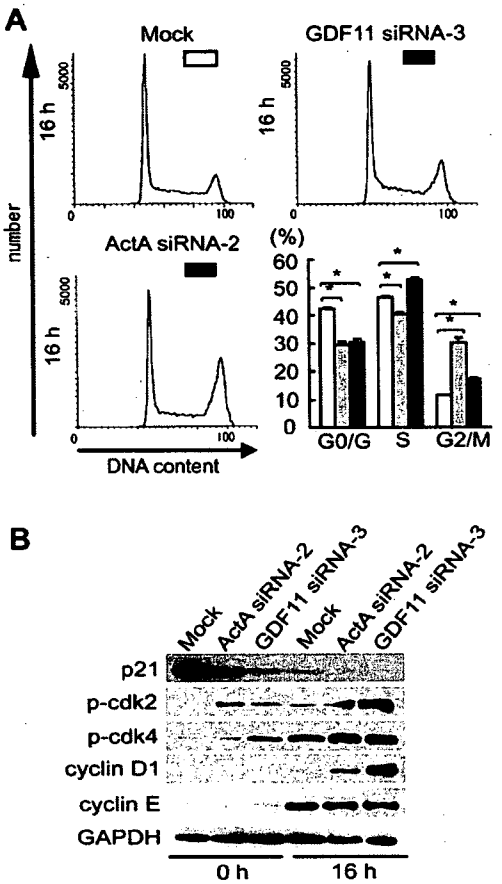

Fig. 4. Knockdown of ActA and GDF11 accelerates cell cycle progression through down-regulation of p21, activation of cdk2/4, and cyclin D1 induction in MDPCs. (A) At the start of the experiment, MDPCs were infected with Mock, ActA, or GDF11 siRNA. After 24 h in serum-free conditions, the medium was changed to growth medium and the cells were cultivated for 16 h before being subjected to FACS. Mean values ± SE of 5 independent experiments are shown. $^{*}p < 0.01$. (B) Equal amounts of protein from MDPCs obtained from the cells infected as indicated were used to evaluate the expression of cell cycle regulators by Western blotting. Cell cycle arrest was induced by serum-free culturing followed by growth medium stimulation for 16 h. The results shown are representative blots obtained from 5 independent experiments.

respectively, entered in S phase (Fig. 4A, $p < 0.01$). Analysis of the cell cycle distribution at 16 h in ActA knockdown MDPCs showed that the cells had been driven into G2/M phase (30.2 ± 1.6%), whereas only a small control-cell population was present in mitotic phase (Fig. 4A; 11.4 ± 0.3%, $p < 0.01$).

Given that endogenous ActA and GDF11 regulate the process of cell cycle withdrawal of MDPCs, we speculated that these ligands may affect the cell-cycle-regulatory proteins. To examine this possibility, actively proliferating MDPCs infected with Mock or siRNA of ActA or GDF11 were cultured in growth medium for 16 h. Before serum stimulation (0 h), ActA and GDF11 siRNA-infected MDPCs demonstrated down-regulation of the p21 levels coincident with increased activity of cdk2/4 (Fig. 4B). These changes were significantly enhanced when the MDPCs were treated with serum for 16 h. In addition, undetectable or low levels of cyclin D1/E were present in MDPCs prior to the serum stimulation, and following exposure to serum for 16 h, the protein level of cyclin D1 was significantly increased in MDPCs with ActA or GDF11 knockdown (Fig. 4B).

Discussion

The enhancement of muscle regeneration by specific growth factors has been reported [8,9]; however, factors that might activate muscle-derived cardiogenic progenitor cells remain unknown. Previous studies have clearly shown that myosin light chain promoter-driven transgenic mice of follistatin or a dominant-negative form of ActRII and *mstn*−/− mice exhibited large increases in muscle mass

through satellite cell proliferation [12,15]. However, the absence of myostatin expression in myospheres highlighted the distinctness of their phenotype from that of satellite cells and also suggested that endogenous ActA and GDF11 appeared to be the plausible candidates to negatively regulate MDPC self-replication through Smad2/3 phosphorylation, which has been observed in ES cells [17].

As previously shown in other cell types, follistatin has been reported to activate hepatic satellite cells and neural progenitors through inhibiting the action of ActA and GDF11 [11,18]. Endogenously expressed ActA and/or GDF11 in myospheres, both signaling can be inhibited by follistatin, may act as negative regulators of the cardiogenic progenitor cell number, whereas follistatin can also induce myoblast fusion through neutralizing myostatin [19].

Our data demonstrated for the first time that follistatin induced cell cycle progression of MDPCs through inhibition of ActA and GDF11 signaling. Inhibition of the endogenous expression of ActA and GDF11 in MDPCs may increase cyclin D1 levels through Smad2/3 inactivation, which is in contrast to the recent report showing that exogenous myostatin signaling promoted cyclin D1 degradation independently of the Smad3 pathway in C_2C_{12} cells [20]. Myostatin is known to repress myogenic cell proliferation through up-regulation of p21 expression and inhibition of cdk2 activity [21]. The lack of alteration of cdk4 and cyclin D1 protein levels by myostatin treatment in C_2C_{12} cells highlighted the existence of distinct signaling pathway mediated through activin and GDF11 in MDPCs [22]. Our results suggest that follistatin may be an effective reagent to promote muscle-derived cardiogenic progenitor cell growth ex vivo for application in regenerative medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.087.

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Abstract—The role of Smads and their specific ligands during cardiomyogenesis in ES cells was examined. Smad2 was activated bimodally in the early and late phases of cardiac differentiation, whereas Smad1 was activated after the middle phase. Nodal and Cripto were expressed in the early stage and then downregulated, whereas transforming growth factor-β and activin were expressed only in the late phase. Suppression of early Smad2 activation by SB-431542 produced complete inhibition of endodermal and mesodermal induction but augmented neuroectodermal differentiation, followed by poor cardiomyogenesis, whereas inhibition during the late phase alone promoted cardiomyogenesis. Inhibitory effect of Smad2 on cardiomyogenesis in the late phase was mainly mediated by transforming growth factor-β, and inhibition of transforming growth factor-β–mediated Smad2 activation resulted in a greater replicative potential in differentiated cardiac myocytes and enhanced differentiation of nonmyocytes into cardiac myocytes. Thus, endogenous Smad2 activation is indispensable for endodermal and mesodermal induction in the early phase. In the late phase, endogenous transforming growth factor-β negatively regulates cardiomyogenesis through Smad2 activation by modulating proliferation and differentiation of cardiac myocytes. (***Circ Res.*** **2007;101:78-87.)**

Key Words: embryonic stem cells ■ cardiomyogenesis ■ Smad2 ■ TGF-β ■ differentiation

Embryonic stem (ES) cells are well-established pluripotent stem cells and capable of self-renewal and differentiation into derivatives of all 3 primary germ layers. With appropriate culture conditions, ES cells can differentiate into specialized cells including cardiac myocytes in vitro. The in vitro differentiation of ES cells into cardiac myocytes not only provides unique opportunities to study development of cardiac myocytes but also proposes the potential use of ES cell–derived cardiac myocytes for many medical applications such as cell transplantation therapy against various heart diseases and pharmacological testing on cardiac myocytes.[1] However, the molecular mechanisms governing differentiation of ES cells into specific lineages are poorly understood, and their comprehension would improve the efficiency of differentiation into specific cell types.

Members of transforming growth factor (TGF)-β superfamily are pleiotropic cytokines involved in many biological processes and signaling via heteromeric complexes of type I and type II serine/threonine kinase receptors.[2] On ligand binding and heterodimerization, the constitutively active type II receptor kinase phosphorylates the type I receptor, which in turn activates downstream signal transduction cascades including Smad pathways. According to the usage of different sets of type I receptors and receptor-regulated Smad (R-Smad), the superfamily members can be classified into 2 major branches: (1) the TGF-β/activin/Nodal branch and (2) the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) branch.[2] The TGF-β/activin/Nodal branch activates activin receptor–like kinase (ALK)-4, -5, and -7, which phosphorylate Smad2 and -3, whereas Smad1, -5, and -8 are substrates for the BMP/GDF branch through ALK-1, -2, -3, and -6.[2,3] The role of Smad1/5/8-activating BMP in cardiogenesis is relatively well documented. In chick embryo, BMP2 is able to induce expression of myocardial lineage markers in ectopic locations in vivo, and anterior lateral mesoderm explant cultures in vitro,[4] and Noggin, one of soluble BMP antagonists, prevents myocardial differentiation of lateral mesendoderm culture in vitro.[4] The dependence of myocardial specification on BMP signaling is evolutionally conserved, although several members of BMP have overlapping function in murine cardiac differentiation.[5] In murine teratocarcinoma P19CL6 cells, myocardial differentiation is shown to depend on functional BMP signals,[6] supporting the essential role for BMP signaling in cardiac specification. However, little is yet known about the function of Samd2/3-activating TGF-β, activin, and Nodal in cardiac differentiation.

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